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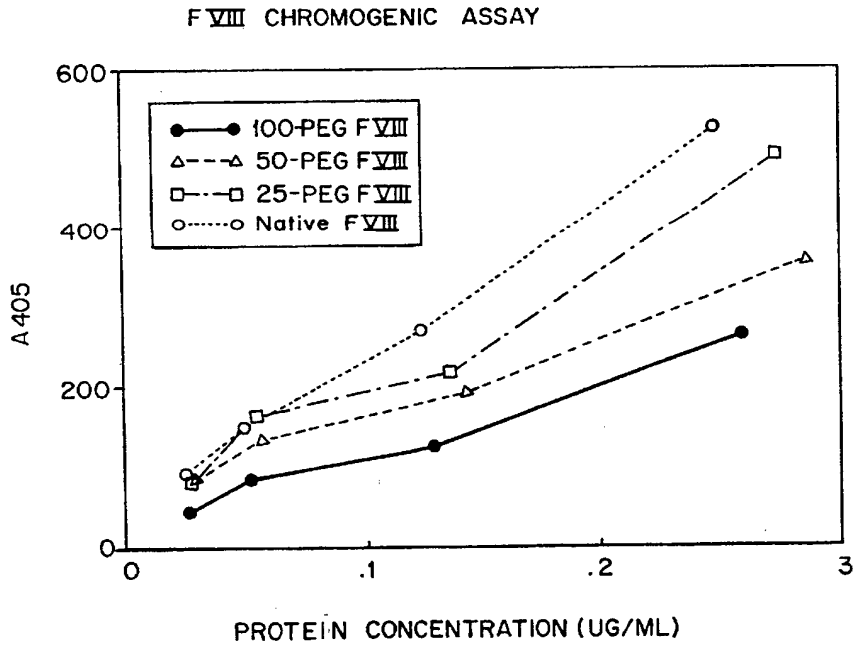
(57) Abstract

Conjugates containing a substance with coagulant activity, such as recombinant factor VIII, and non-antigenic polymers, such as poly(ethylene glycol), are disclosed (as shown in the figure). Also disclosed are methods of forming the novel conjugates of this invention.



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FACTOR VIII - POLYMERIC CONJUGATE

The present invention is directed to methods of modifying substances having Factor VIII activity, such as the pro-coagulant glycoprotein Factor VIII and, more particularly, to methods for the modification of such substances with non-antigenic polymers, such as polyethylene glycol, and the resultant conjugates.

Background of the Invention

The process of blood clotting typically begins with trauma to a blood vessel. The damaged vessel wall initiates hemostasis by causing adherence and accumulation of platelets at the injury site and by activating plasma proteins which initiate the coagulation process. A series of proteins, including Factor VIII, are activated sequentially by specific proteolytic cleavages and conformational changes leading to the deposition of insoluble fibrin which curtails the blood flow.

In addition to forming a platelet plug, the platelets release vasoactive amines which cause arteriolar vasoconstriction causing locally reduced blood pressure that lasts for hours. This, in combination with the platelet plug, effectively controls bleeding.

Hemophilia is a bleeding disorder caused by the lack of an essential blood factor. Hemophilia A (classic hemophilia) is the most common and is caused by a genetic deficiency or abnormality of Factor VIII. Hemophilia B

(Christmas disease) is caused by a deficiency or abnormality of Factor IX. Hemophilia is an X-linked genetic abnormality and therefore primarily affects males. Approximately 10-20/100,000 males have hemophilia A and 1/100,000 have hemophilia B.

Hemophilia is a heterogeneous disorder whose severity of bleeding tendency is determined by the specific point mutations. Mutant Factor VIII proteins can have clotting activities that vary from near normal to severely deficient. The clinical features of hemophilia A and B are identical. Less than 1% clotting factor activity is defined as severe hemophilia, and is accompanied by spontaneous bleeding in the muscles and larger joints. Repeated bleeding in joints causes arthropathy which is a major chronic complication. Hemarthroses are worsened by muscle atrophy due to muscle bleedings. This affects both posture and gait of the older patient. In about one-half of all hemophiliacs, repeated hemarthroses result in eventual deformity and crippling. These patients usually have Factor VIII levels of <5% of normal. In mild hemophilia (5-40% FVIII or FIX), bleeding does not occur except after trauma. Moderately severe hemophilia (1-5% FVIII or FIX) has clinical features between the severe and mild hemophilia.

Conventional treatment of hemophilia consists of replacement of the Factor VIII from pooled donor plasm including fresh, frozen plasma or Factor VIII concentrates and, more recently, recombinant Factor VIII products. Home therapy, surgery and prolonged treatment of hemophiliacs have been eased by the availability of Factor VIII

preparations. With the availability of replacement blood factors, the life expectancy for hemophiliacs is almost normal. However with the increased use of pooled donor preparations, there has been a higher transmission of viral diseases such as hepatitis (eg. B, delta, non A non B) and HIV. It has been estimated that 50% of hemophilia patients are either hepatitis positive or HIV positive. These viral infections are now the major cause of morbidity and mortality in patients with hemophilia.

To decrease the possibility of transmitting viral infections through blood factor preparations, firms have increased purification techniques to reduce the virus load. These measures can include pasteurization, detergent/solvent disruption of viral membranes, and monoclonal antibody purification. Blood banks now routinely screen all donor blood for AIDS virus and hepatitis. Yet none of these measures can guarantee freedom from viral contamination.

Further disadvantages of pooled donor plasma include the cost and availability of the purified blood factors. With increased purification steps, the cost of blood factor therapy has increased. Availability of the blood factors is also a concern. Theoretically, these factors should be administered prophylactically in many cases to avoid the sequelae of uncontrolled bleeding (eg. development of joint disorders). However, cost, availability and the pharmacokinetics make an effective prophylactic therapy unfeasible.

With the advent of recombinant DNA technology, researchers have now cloned and are testing a number of blood factors, including rh-factor VIII:c in patients with hemophilia. While recombinant technology may overcome the problems of viral contamination and availability, it does not affect the pharmacokinetics of the factors nor the formation of inhibitors (antibodies) in patients. It is estimated that 10-15% of all patients with hemophilia A will develop IgG antibodies that will nullify the value of replacement therapy. Inhibitor development occurs primarily in patients with severe hemophilia although antibodies to Factor VIII in mild hemophilia have been reported. Approximately 5-15% of patients with severe hemophilia have antibodies to Factor VIII or IX. It has been estimated that the actual risk of developing neutralizing antibodies by age 20 is as high as 15-24%. Joint bleedings often cannot be controlled and adequately treated and many of these patients are severely handicapped.

To overcome the neutralizing effect of the antibodies, physicians can be forced to increase the dosage to the factor. However, there is often a decreased response to the replacement therapy despite increases in dosage. Care in administering the factors as well as the administration of steroids and other immunosuppressive agents such as azathioprine, cyclophosphamide and high-dose i.v. gammaglobulin G is often required to prevent or limit the development of antibodies and hypersensitivity reactions. Antibody depletion through plasmapheresis has been used to decrease the inhibitor titers in circulation. Interferon α2a has been also used successfully to treat

cases of postpartum acquired inhibition to Factor VIII:c. However, such immunosuppressive techniques have been only partially successful and raise the risk that the patient will be more vulnerable to opportunistic infections, eg. HIV or hepatitis.

In light of the complications and risks inherent in the conventional treatment of classic hemophilia, it is desirable to provide a modified Factor VIII which is less likely to cause the formation of inhibitor antibodies. In light of the high costs of recombinant Factor VIII, it is also highly desirable to increase the clearance time for Factor VIII activity. The terms "disappearance time" and "clearance time" are used herein to denote the time taken for Factor VIII activity to decrease to 50% of its maximum level.

Summary of the Invention

The various embodiments of the present invention provide Factor VIII conjugates having prolonged circulating life and activity in mammals. In addition, there are also provided methods for the modification of Factor VIII with non-antigenic polymeric materials such as, polyethylene glycol (PEG). The Factor VIII fraction included in the conjugates preferably comprises a protein having Factor VIII activity which has been formed using recombinant technology. The Factor VIII fraction may also be derived from human or animal plasma sources, such as bovine or porcine plasma. Transgenic sources are also contemplated. As used herein, the term "Factor VIII fraction" means any substance which demonstrates the ability in vivo to

function as mammalian Factor VIII, i.e. activate Factor X and continue the intrinsic clotting cascade. The Factor VIII fraction can also comprise other proteins and reagents such as Von Willebrands' factor, as well as other serum proteins including albumin, fibrin, fibrinogen, etc.

According to one embodiment of the present invention, Factor VIII preparations are reacted with molar excesses of a suitable activated polyalkalene oxide such as methoxypoly(ethylene glycol)-N-succinimidyl carbonate (SC-PEG) under conditions sufficient to effect conjugation while maintaining at least a portion of the Factor VIII activity. Such conditions include reacting the substituents at temperatures of up to about 27°C and in pharmaceutically acceptable buffer systems. As used herein, the term "molar excess" is meant to indicate the ratio of the number of moles of polymeric substance to the number of moles of Factor VIII. The reaction is then terminated by adding a molar excess of a compound that reacts very quickly with any free SC-PEG, such as glycine. The resulting modified Factor VIII may then be advantageously stabilized with human serum albumin (HSA) and sterile filtered. This method has been found to provide a long acting modified Factor VIII conjugate which is less susceptible to antibody inhibitors and retains a large percentage of the protein's original activity. The conjugates are substantially resistant to <u>in vivo</u> hydrolysis and thus provide prolonged activity after administration.

These and other embodiments of the present invention are described in detail below.

Brief Description of the Drawings

Figure 1 is a graph indicating the activity of a PEG-modified Factor VIII, prepared according to one embodiment of the present invention, as a function of total protein concentration.

Detailed Description

The various embodiments of the present invention advantageously provide methods for modifying substances having Factor VIII activity with substantially non-antigenic polymeric substances under relatively mild conditions, for example at a pH of about 6.5 - 8, preferably of about 6.5 - 7.5, and at temperatures which do not exceed 27°C, and are preferably in the range of from 2 - 10°C. Those skilled in the art will appreciate that Factor VIII is a relatively large and sensitive glycoprotein. The present invention provides methods for modifying Factor VIII without subjecting the protein to harsh conditions which could eliminate its activity.

The substantially non-antigenic polymer substances included in the conjugates are preferably poly(alkylene oxides). Within this group of substances are alpha-substituted polyalkylene oxide derivatives such as methoxypolyethylene glycols or other suitable alkyl-substituted derivatives such as C_1-C_4 alkyl groups. It is preferred, however, that the non-antigenic material be a monomethyl-substituted PEG homopolymer. Alternative polymers such as other polyethylene glycol homopolymers,

polypropylene glycol homopolymers, other alkyl-polyethylene oxides, bis-polyethylene oxides and co-polymers or block co-polymers of poly(alkylene oxides) are also useful. In those aspects of the invention where PEG-based polymers are used, it is preferred that they have molecular weights of from about 1,000 to about 10,000. Molecular weights of about 2,000 to 7,000 are preferred and molecular weights of about 5,000 are particularly preferred.

As stated above, covalent modification of the protein material is preferred to provide a hydrolysis-resistant conjugate. The covalent modification reaction includes reacting a substance having the desired Factor VIII activity with a substantially non-antigenic polymeric substance under conditions sufficient to effect conjugations while maintaining at least a portion of the Factor VIII activity.

The polymers may be activated in order to effect the desired linkage with the protein substance. By activation, it is understood by those of ordinary skill in the art that the polymer is functionalized to include a desired reactive group. Examples of such activation are disclosed in U.S. patents 4,179,337 and 5,122,614, which are hereby incorporated by reference. In the disclosures of these patents, the hydroxyl end groups of polyalkylene glycols are converted into reactive functional groups and thus activated.

According to one preferred embodiment, a Factor VIII fraction is modified with SC-PEG such as disclosed in the '614 patent, supra. This particularly preferred

activated form of PEG for use in the present invention is poly(ethylene glycol)-N-succinimide carbonate. This activated polymer forms stable, hydrolysis-resistant carbamate (urethane) linkages with amino groups of the protein. Isocyanate-activated PEG's are also of use. While the references incorporated herein describe epsilon amino group modifications of lysine, other conjugation methods are also contemplated. Carbohydrate and/or acid group or other amino acid modifications are also within the scope of the present invention. Covalent linkage by any atom between the protein and polymer is possible. Moreover, non-covalent conjugation such as lipophilic or hydrophilic interactions are also contemplated.

In order to prepare the Factor VIII fraction for the polymeric modification, the pH of the Factor VIII fraction is preferably adjusted to about 6.5 - 8, most preferably to about 6.5 - 7.5. The Factor VIII may be adversely affected by a pH above about 8 so that range should be avoided. The pH of the Factor VIII fraction is preferably modified through a vigorous buffer exchange by dialyzing the Factor VIII against an appropriate salt buffer system. For example, the buffer exchange can be conducted by placing the Factor VIII fraction in a dialysis bag suspended in a salt buffer and changing the buffer with fresh solution several times. The salt buffer may, for example, comprise 50 mM sodium phosphate and 100 mM sodium chloride per liter at a pH of 7. Such a buffer exchange is also useful in removing undesirable low molecular weight components which may be present in commercially available Factor VIII fractions.

The process of the present invention includes preparing or providing the activated polymer and, thereafter, reacting it with the Factor VIII substance. A reaction mixture is prepared by adding a solution containing the activated polymer, preferably SC-PEG, to the Factor VIII fraction. The SC-PEG solution is preferably prepared with the same buffer and pH, i.e. 6.5 - 8, as the salt buffer utilized in the buffer exchange. In the reaction mixture, the protein is reacted with an appropriate amount of the activated polymer, which is typically present in a several-fold molar excess over the enzymatic-like substance. The polymeric excess will range from about 5 to about 125 fold molar excess and preferably from about 15 to about 50 fold molar excess of the polymer to the Factor VIII protein. The reaction is carried out at temperatures of from about 2 to 27°C, and preferably at temperatures of from 2 - 10°C over time periods ranging from a few minutes to as long as 12 hours. Depending upon the reaction conditions, the artisan can tailor the profile of the resultant conjugate. For example, large molar excesses of polymer reacted with the protein result in conjugates having relatively long circulating times in vivo but somewhat less activity than unmodified or slightly modified proteins. The inverse of the foregoing is also true. Smaller molar excesses reacted with the protein provide conjugates with higher activity and somewhat shorter circulating life. In all instances, the conjugate has significantly prolonged circulating life in vivo over the unmodified protein.

One of the advantages of the present modification process is that it can be carried out at relatively mild

reaction conditions which will not adversely effect the protein. The reaction is then stopped by adding a molar excess of a compound which reacts quickly with the SC-PEG. For example, a 250-fold molar excess of glycine is sufficient to terminate the reaction between the SC-PEG and the Factor VIII.

Following the conjugation reaction, the desired product is recovered using known techniques and purified using column chromatography or similar apparatus if necessary. For example, excess reagents can then be removed from the reaction mixture by the same dialysis procedure described above. The modified-Factor VIII fraction is then preferably formulated with a stabilizer. For example, human serum albumin (HSA), which acts as a carrier protein and protects the modified Factor VIII from proteolytic cleavages. A final concentration of about 0.5 mg/ml to about 5 mg/ml HSA, e.g. about 1 mg/ml HSA, and about 0.5 mg/ml to about 5 mg/ml, e.g. about 1 mg/ml, of modified Factor VIII is suitable. The stabilized modified-Factor VIII is then preferably gravity filtered through a sterile filter, e.g. a 0.45 micron filter. Gravity filtration is preferred to other forms of filtration, such as syringe filtration, since Factor VIII is a shear sensitive molecule whose activity can be adversely affected by the shear forces encountered during a more rigorous filtration.

After stabilization and filtration, the modified-Factor VIII may also be lyophilized. Tests have shown that Factor VIII modified by the above process have retained large percentages of their activity even after being

lyophilized and reconstituted.

Another aspect of the present invention provides methods of treatment for hemophilia. The method includes administering, in a pharmaceutically acceptable vehicle, such as a parenteral solution, an effective amount of the compositions described herein to alleviate clotting deficiencies. Those of ordinary skill in the art will realize that the amount of the conjugate used in the method of the present invention will vary somewhat from patient to patient, however, conjugates capable of delivering from about 15 IU/kg to about 100 IU/kg per administration or an amount sufficient to maintain a level greater than 0.01 IU/ml blood are preferred. The optimal dosing of the conjugate can be determined from clinical experience.

Example 1

Modification of Factor VIII with Polyethylene Glycol (PEG) Purified Factor VIII (5 mg), obtained from Alpha Therapeutics, Los Angeles, CA, was dialyzed against 50 mM sodium phosphate pH 7.0, 100 mM NaCl (4 X 1L) overnight. To four 1 mg aliquots was added a 0, 25, 50 and 100-fold molar excesses of methoxypoly(ethylene glycol)-N-succinimidyl carbonate (SC-PEG), respectively. The SC-PEG was added as a 10 mg/ml solution in the above buffer and the reaction mixtures were allowed to set on ice for 2 hours with occasional stirring, at which time the reactions were stopped by the addition of a 250-fold molar excess of glycine. Excess reagents were then removed by dialysis as described above.

Factor VIII activity was determined using a chromogenic assay. Even after treatment with up to 100-fold excess SC-PEG, 45% of the original activity remained as illustrated in Figure 1. Figure 1 is a graph of the activities, measured by a DADE Factor VIII chromogenic assay kit (A_{405}) obtained from Baxter Healthcare, Deerfield, Illinois, of the modified Factor VIII proteins formed in Example 1. As indicated in the legend, the control "Native Factor VIII" which underwent all reaction steps without any SC-PEG had the highest activity. This graph indicates that high activities are retained by the Factor VIII even after extensive modification with SC-PEG.

Example 2

Reaction mixtures containing 0, 25, and 50-fold molar excesses of SC-PEG, respectively, with Factor VIII fractions were prepared as above but were then formulated with 1 mg/ml HSA prior to gravity filtration through a 0.45 micron sterile filter. Chromogenic assays of filtered samples gave the following results:

Sample	SPECIFIC ACTIVITY U/mg F.VIII	% CONTROL ACTIVITY
0-PEG	44.9	100%
25-PEG	40.7	91%
50-PEG	43.7	97%

Aliquots (1 ml) of the samples were then lyophilized and vacuum sealed in serum vials using an FTS shelf lyophilizer. The samples were then reconstituted with 1 ml water and compared with the unlyophilized

controls in the chromogenic assay. Activity before and after lyophilization were as follows:

Sample	SPECIFIC ACTIVITY U/mg F.VIII		% CONTROL ACTIVITY
Before	After		
0-PEG	44.9	23.4	52%
25-PEG	40.7	16.4	36%
50-PEG	43.7	35.5	79%

Example 3

Factor VIII was modified in this example with poly(ethylene glycol) succinoyl-N-hydroxysuccinimide ester (SS-PEG). The Factor VIII was dialyzed against 50 mM sodium phosphate pH 7.0, 100 mM NaCl overnight. Three, 500 mg reactions were set up with 25, 50 and 100 fold excesses, respectively, of SS-PEG. The SS-PEG was added as a 10 mg/ml solution in the above buffer. The reactions proceeded on ice for 2 hours with occasional stirring and were then quenched by the addition of a 100 fold excess of glycine. HSA was added to 5% (w/w) and all samples were dialyzed overnight in 59 mM sodium phosphate pH 7.0, 100 mM NaCl. The results of the chromogenic assay for Factor VIII activity were as follows.

SAMPLE	% CONTROL ACTIVITY
UNMODIFIED F. VIII	100
25-PEG F. VIII	68
50-PEG F. VIII	52
100-PEG F. VIII	35

Example 4

The circulating half-life of PEG-modified Factor VIII of the present invention was determined. Three mice were injected in the tail vein with 100 Units of Factor VIII obtained from Alpha-Therapeutics and sets of three mice each were similarly injected with PEG-Factor VIII (SC-PEG) prepared in the manner described above. The mouse plasma was collected after 4 hours, 8 hours, 24 hours, 48 hours, 72 hours and 7 days via orbital eye bleed. At the indicated times, plasma was obtained from the mice and stored at 4°C until assay. Pooled plasma samples from each time point were assayed for clotting activity using the Factor VIII chromogenic assay. The data was graphed and the $T_{½}$'s were calculated from the slopes.

The average $T_{½}$ for the samples is given in the following table. Each of the PEG-modified Factor VIII samples had a significantly longer $T_{½}$ than did the unmodified Factor VIII control. Additionally, the PEG-Factor VIII preparations were absorbed much more rapidly into the blood stream than the unmodified Factor VIII.

HALF-LIVES OF FACTOR-VIII IN MICE

COMPOUND	ACTIVITY	$T_{½}$
Unmodified Factor VIII	100%	13 hrs.
PEG-Factor $VIII_{25}$	81%	31 hrs.
PEG-Factor $VIII_{50}$	88%	56 hrs.
PEG-Factor $VIII_{100}$	79%	56 hrs.

The various embodiments of the present invention, therefore, provide conjugates which retain significant levels of Factor VIII activity while increasing circulating half-life and having less of a tendency to cause the formation of inhibitor antibodies.

WE CLAIM:

1. A biologically active conjugate comprising a first substance having Factor VIII activity bound to a substantially non-antigenic polymeric substance with a carbamate (urethane) linkage.

2. The conjugate of claim 1 wherein said first substance comprises factor VIII.

3. The conjugate of claim 2 wherein said factor VIII is of recombinant origin.

4. The conjugate of claim 2 wherein said factor VIII is of mammalian origin.

5. The conjugate of claim 2 wherein said factor VIII is of transgenic origin.

6. The conjugate of claim 1 wherein said first substance comprises fractions of the protein Factor VIII.

7. The conjugate of claim 1 wherein said polymeric substance comprises a poly(alkylene oxide).

8. The conjugate of claim 7 wherein said polymeric substance comprises an alpha-substituted polyalkylene oxide derivative.

9. The conjugate of claim 7 wherein said polymeric substance is selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol

homopolymers, alkyl-capped polyethylene oxides, bis-polyethylene oxides and copolymers or block copolymers of poly(alkylene oxides).

10. The conjugate of claim 7 wherein said polymeric substance comprises poly(ethylene glycol).

11. The conjugate of claim 7 wherein said polymeric substance has a molecular weight of about 1,000 to about 10,000.

12. The conjugate of claim 11 wherein said polymeric substance has a molecular weight of about 2,000 to about 7,500.

13. The conjugate of claim 11 wherein said polymeric substance has a molecular weight of about 5,000.

14. A method of preparing a conjugate having Factor VIII activity comprising reacting a first substance having Factor VIII activity with a substantially non-antigenic polymeric substance under conditions sufficient to effect conjugation of said first substance and said polymeric substance with a carbamate (urethane) linkage while maintaining at least a portion of the activity of the first substance.

15. The method of claim 14 wherein said polymer is a poly(alkylene oxide).

16. The method of claim 14 wherein said

polyalkylene oxide is an alpha-substituted polyalkylene oxide derivative.

17. The method of claim 16 wherein said poly(alkylene oxide) is a polyethylene glycol.

18. The method of Claim 14 wherein said reacting step comprises providing a molar excess of said substantially non-antigenic polymeric substance relative to said first substance.

19. The method of Claim 18 wherein said reacting step comprises providing about a 15 - 50 fold molar excess of said substantially non-antigenic polymeric substance relative to said first substance.

20. The method of Claim 14 wherein said reacting step is conducted at temperatures of up to about 27°C.

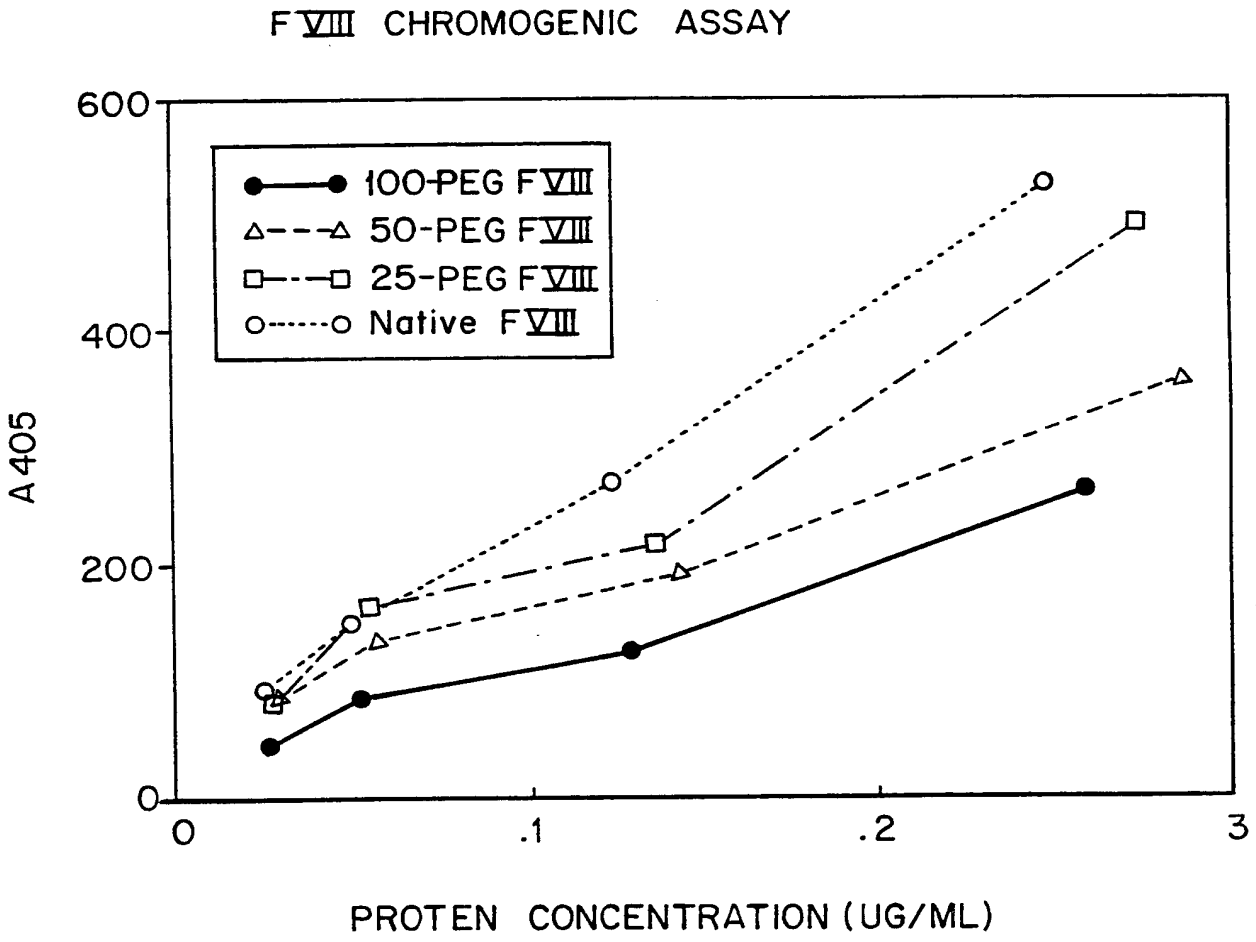
21. The method of Claim 20 wherein said reacting step is conducted at temperatures of about 2 - 10°C.

22. The method of Claim 14 wherein said first substance comprises Factor VIII.

23. A method of treating hemophilia comprising administering a therapeutically effective amount of the conjugate of claim 7.

24. A method of treating hemophilia comprising administering a therapeutically effective amount of the conjugate of claim 1.

FIG. 1


FVIII CHROMOGENIC ASSAY
600
400
200
0
A405
100-PEG FVIII
50-PEG FVIII
25-PEG FVIII
Native FVIII
0
.1
.2
3
PROTEN CONCENTRATION (UG/ML)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00552

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 35/14;C08G 71/00;C07K 3/06
US CL :530/383,402,406;514/12,21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/383,402,406;514/12,21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS online, medline, aps, biosis,embase,derwent
search terms:Factor VIII, conjugates,urethane, carbamate,polyethyleneglycol, polyalkylene oxide.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US,A, 4,970,300 (Fulton et al.) 13 November 1990, col. 11, claims 1, 2, 5-7, 9.	1-18, 20-24 ---------- 19
Y	WO,A, 92/16555 (Enzon, Inc.) 01 October 1992, pages 8, 11, 13, lines 33-38, claims 1-2, 5, 7, 10-16, 24, page 1, lines 15-17.	1-24
Y	US, A, 5,122,614 (Zalipsky) 16 June 1992, col. 1, 2 and 3, FIGS. 1-3.	1-24
Y	US, A, 5,219,564 (Zalipsky) 15 June 1993,whole document.	1-24
Y,	US, A, 5,234,903 (Nho et al.) 10 August 1993, whole document.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search 04 March 1994	Date of mailing of the international search report MAR 21 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer C. SAYALA Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00552

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Acta Medica et Biologica, vol. 36, number 1, issued 1988, Sakuragawa et al., ""Studies on the Stability of Factor VIII modified by polyethylene glycol", pages 1-5.	1-24